Optimizing T4 DNA polymerase conditions enhances the efficiency of one-step sequence- and ligation-independent cloning

Mohammad Nazrul Islam^{1,2,3}, Kyeong Won Lee¹, Hyung-Soon Yim^{1,2}, Seong Hyuk Lee¹, Hae Chang Jung^{1,2}, Jung-Hyun Lee^{1,2}, and Jae-Yeon Jeong¹

¹Marine Biotechnology Research Center, Korea Institute of Ocean Science and Technology, Ansan, Republic of Korea, ²Department of Marine Biotechnology, Korea University of Science and Technology, Daejeon, Republic of Korea, and ³Department of Biotechnology, Sher-e-Bangla Agricultural University, Dhaka, Bangladesh

BioTechniques 63:125-130 (September 2017) doi 10.2144/000114588

Keywords: seamless cloning; SLIC; T4 DNA polymerase

Supplementary material for this article is available at www.BioTechniques.com/article/114588.

Previously, we developed a one-step sequence- and ligation-independent cloning (SLIC) method that is simple, fast, and cost-effective. However, although one-step SLIC generally works well, its cloning efficiency is occasionally poor, potentially due to formation of stable secondary structures within the single-stranded DNA (ssDNA) region generated by T4 DNA polymerase during the 2.5 min treatment at room temperature. To overcome this problem, we developed a modified thermo-regulated one-step SLIC approach by testing shorter T4 DNA polymerase treatment durations (5 s–2.5 min) over a wide range of temperatures (25–75°C). The highest cloning efficiency resulted when inserts with homology lengths <20 bases were treated with T4 DNA polymerase for 30 s at 50°C. This briefer T4 polymerase treatment at a higher temperature helps increase cloning efficiency for inserts with strong secondary structures at their ends, increasing the utility of one-step SLIC for the cloning of short fragments.

Seamless cloning methods that do not require restriction enzyme digestion of the insert have been developed for high-throughput cloning and synthetic biology. These methods

are divided into three categories: exonuclease activity-based methods (1–3), PCR-based methods (4,5), and ligase-based methods (6). Exonuclease activity-based methods were

developed based on the early ligation-independent cloning (LIC) method (7), which utilizes the exonuclease activity of T4 DNA polymerase to generate single-stranded DNA (ssDNA) regions at the ends of a linearized vector and a PCR-amplified insert, followed by complementary annealing between the ssDNA homology regions of the pre-defined ends of the vector and insert.

Sequence- and ligation-independent cloning (SLIC) (1) and Gibson assembly (2) overcome sequence constraints of LIC and allow for the simultaneous assembly of multiple fragments. Many commercial kits, including In-Fusion (Clontech, Mountain View, CA) and CloneEZ cloning (GenScript, Piscataway, NJ), have been developed based on the same principles as SLIC (1). Although highly efficient, these methods require additional enzymes, such as RecA for SLIC (1), *Taq* ligase for Gibson Assembly (2), or proprietary enzymes for commercial kits. PCR-based methods include circular polymerase extension cloning (4) and overlap extension PCR cloning (5). In PCR-based approaches, no exonuclease-mediated chew-back step takes place, and annealing between the vector and insert occurs at higher temperatures compared with exonuclease-based methods; therefore, PCR-based methods may be most suitable for the cloning of small inserts or inserts with strong secondary structures at the ends. However, unlike exonuclease-based methods, PCR-based methods do have an increased probability of the inserts mis-annealing at other sequences in the vector after the denaturation step and the generation of PCR-derived mutations.

Ligase-cycling reaction cloning starts with a blunt-ended vector and insert, along with single-stranded bridging oligonucleotides; DNA assembly occurs through multiple rounds of denaturation, annealing, and ligation (6). Although useful for

METHOD SUMMARY

One-step sequence- and ligation-independent cloning (SLIC) efficiency was optimized for difficult-to-clone inserts by testing shorter durations of T4 DNA polymerase treatment of the insert and linearized vector over a broad range of incubation temperatures. The highest cloning efficiencies were found with treatment for 30 s at 50°C.

Figure 1. Effect of the duration of T4 polymerase treatment on one-step sequence- and ligation-independent cloning (SLIC). (A,B) Partial sequences of vector pGEX-5X (A) and insert (B). The vector was linearized by *Bam*HI digestion, and the insert was prepared by PCR with the primers shown in (B). Vector homology regions are shown in blue for the forward primer and in cyan or red for the reverse primer. For seamless cloning of *FGF2* immediately after the factor Xa cleavage site, the *Bam*HI site was not included in the forward primer. (C) After mixing the linearized vector and insert in the same tube at room temperature, T4 DNA polymerase was added, and the tubes were immediately incubated at either 25°C or 37°C for the indicated times. The reaction was stopped by putting the tubes on ice. Data represent mean $\pm$ SD ($n = 4$). Colonies were analyzed by restriction enzyme digestion.

BVC FLUID ASPIRATION SYSTEMS

Compact, powerful and quiet solutions for cell culture work

- **Power & Control:** Suitable for two users
- **Robust:** Models designed for in-bottle bleach decontamination
- **Safe:** Self contained systems for collection and disposal of biological waste, plus 0.2 μ m biofilter

Special promotions at www.brandtech.com

Lab Rats Trust BrandTech®!

BRANDTECH
SCIENTIFIC, INC.

888-522-2726 | www.brandtech.com

high-throughput assembly, this method requires optimization.

One-step SLIC is a simple, cost-efficient method (3) with a cloning efficiency comparable to other approaches (8). In one-step SLIC, the linearized vector and PCR-amplified inserts are mixed in a single tube and treated with T4 DNA polymerase at room temperature for 2.5 min. The reaction is stopped by incubating the mixture on ice, and gaps and overhangs are repaired after the mixture is introduced into competent *Escherichia coli* cells. While one-step SLIC works well with most inserts (3), it can have reduced efficiency at times, possibly due to the formation of stable secondary structures in the ssDNA regions at the ends of the inserts. To solve this problem, we studied the effects of the duration and temperature of T4 DNA polymerase treatment on the efficiency of one-step SLIC.

First, we reduced the duration of T4 DNA polymerase treatment to reduce the length of the single-stranded regions in the homology regions at the ends of the vector and inserts that are exposed by the enzyme. In a previous study, the shortest T4 DNA polymerase treatment time we tested—2.5 min—produced the highest cloning efficiency (3). Therefore, we tested even shorter treatment durations—as low as 5 s—at 25°C or 37°C using the vector pGEX-5X, digested with *Bam*HI, and an insert amplified with primers that have 18-mer and 15-mer homology regions (Figure 1, A and B). Interestingly, the greatest number of colonies was generated when the DNA was treated with T4 DNA polymerase for 30 s at 37°C (Figure 1C). Longer incubations dramatically reduced cloning efficiency, implying that exposing an ssDNA region that exceeds the length of the homology region may interfere with the correct annealing of the vector and insert. Considering that T4 DNA polymerase is a mesophilic enzyme exhibiting 3'-to-5' exonuclease activity with a rate of ~40 bases/min on double-stranded DNA (dsDNA) (9), 30 s at 37°C was sufficient to remove the 18 and 15 nucleotides from the double-stranded homology regions at the ends of the vector and insert. At 25°C, a 1 min T4 DNA polymerase incubation produced

the highest efficiency, suggesting this method requires at least 1 min to fully digest the homology region at this lower temperature.

Next, we examined the effect of temperature on the efficiency of one-step SLIC. T4 DNA polymerase is typically inactivated by incubation at 75°C, but this temperature is also ideal for secondary structure denaturation. If higher temperatures are indeed needed for denaturation, reactions may need to be run longer to compensate for reduced enzyme efficiency. However, higher temperatures and longer incubations may reduce cloning efficiency by interfering with the homology-based annealing of the vector and insert. Taken together, SLIC efficiency depends on a balance of three temperature-dependent factors: (i) sufficient enzyme activity to make a single-stranded region, (ii) denaturation of secondary structures within the exposed single-stranded region, and (iii) inhibition of annealing at high temperatures.

To find the optimum conditions for SLIC, the *PARK7* coding sequence was amplified with the following primers: PARK7-F (5'-**tcgactcgagcggccg**cttc-*caaaagagctctgg*-3') and PARK7-R (5'-**agtcacgatgcggccct**agtctttaagaa-*caagtgg*-3') (bold letters indicate the homology region) and mixed with *Not*I-digested pGEX-5X vector. DNA was treated with T4 DNA polymerase at 25, 37, 50, 65, or 75°C for 15, 30, or 60 s (Figure 2A). The number of colonies increased as the temperature increased up to 50°C and decreased drastically at higher temperatures regardless of the duration of enzyme treatment. The maximum number of colonies was obtained by incubation at 50°C for 30 s. At 25°C, 60 s yielded more colonies than 30 s, implying reduced enzyme activity at this temperature. At 65°C and 75°C, fewer colonies were obtained when DNA was treated for longer periods, suggesting that vector and insert annealing was inhibited by a longer incubation at higher temperatures.

To assess cloning efficiency, four colonies were picked at random from each condition, and their plasmids were analyzed by restriction digestion. When DNA was treated with T4 DNA

Introducing ali-Q™

The first-ever aliquoting pipet controller

One-button aliquoting

Get accurate and precise aliquots without the need to “eyeball” the meniscus.

- Faster aliquoting with the press of one button.
- Also aspirates and dispenses like other pipet controllers.
- Uses any size or brand serological pipet.

Use **VistaLab™ Wobble-not™** serological pipets with ali-Q for the ideal pipetting system.

See the ali-Q™ in action:
vistalab.com/ali-q

VistaLab™
TECHNOLOGIES
Think Differently. Feel Better.

vistalab.com/ali-q • +1 914-244-6226
2 Geneva Road, Brewster NY 10509

polymerase for 30 and 60 s, 100% cloning efficiency was obtained up to 50°C (Figure 2B). Although the number of colonies tested was small, high cloning efficiency was also observed at 65°C. Taken together, these results suggest that secondary structure denaturation increases cloning efficiency up to 50°C, but inhibition of vector and insert annealing reduces cloning efficiency at higher temperatures.

Our results suggest that: (i) 30 s is sufficient to make a single-stranded region with a <20 base homology region at 37°C and higher temperatures, and (ii) 50°C is the highest tested temperature that denatures secondary structures without interfering with annealing between vector and insert.

Recently, Koskela and Frey developed a method termed exonuclease and ligation-independent cloning (ELIC) (10). In ELIC, a linearized vector and insert with homology regions at each end are mixed in water. Competent *E. coli* cells are then transformed directly with the DNA mixture. There is no exonuclease treatment step in this method. To compare the efficiency of SLIC with that of ELIC, we tested the following samples: (i) vector and insert mixed in water at 25°C for 30 s; (ii) vector and insert mixed in T4 DNA polymerase buffer without enzyme at 25°C for 30 s; (iii) vector treated with T4 DNA polymerase in buffer at 25°C for 30 s; (iv) vector and insert mixed with T4 DNA polymerase in buffer and kept on ice (no incubation);

and (v) vector and insert mixed with T4 polymerase in buffer at 50°C for 30 s (Figure 2C). For samples containing both vector and insert (i, ii, iv, and v), the number of colonies observed was similar for T4 DNA polymerase-treated and untreated samples, but positive colonies were found only in samples that were treated with T4 DNA polymerase for 30 s (v), based on restriction digestion analysis of plasmids. When DNA was not treated by T4 DNA polymerase (i, ii, and iv), all of the colonies were self-ligated clones. In contrast, when vector alone was treated with T4 DNA polymerase for 30 s, a significantly smaller number of colonies was obtained (iii). These results imply that cohesive vector ends (four bases) generated by single restriction enzyme digestion are sufficient for self-ligation after transformation in the absence of T4 DNA polymerase treatment but not sufficient for self-ligation after an adjacent region has also been exposed as a single strand by T4 DNA polymerase. These data clearly show that treatment of vector and insert with T4 DNA polymerase is required not only for the generation of a single-stranded region for homology-based annealing but also to reduce self-ligation of a linearized vector, especially by single restriction enzyme cutting, as shown in Figure 2C. It should be noted that one difference between ELIC and SLIC is that in ELIC two different restriction enzymes or blunt-ended vectors are required, as

is subsequent dephosphorylation of the vector (10), but SLIC does not require two different restriction enzymes or dephosphorylation of a vector.

Finally, we tested whether higher temperatures with shorter T4 DNA polymerase treatment times would increase cloning efficiency for difficult-to-clone inserts. The Ton_0858 sequence was PCR-amplified with primers 0858-F (5'-gaccatgattacgaattggatc-cagaagagcgacgatggatcgagga-3') and 0858-R (5'-cggaggtgggatatcccgat-caaatatcacccaaaataataattt-3'), and the pUC118-HMG vector was digested with *Bam*HI (3). After transformation, we observed 2 types of colonies: visible, regular-sized colonies after incubating the plates for 16 h and smaller colonies that appeared after incubating the plates for another 2 h (Figure 3B). The total number of colonies produced by the Ton_0858 transformation was <10% that of the transformations depicted in Figures 1C and 2A. Although the numbers of regular-sized colonies did not change significantly depending on the reaction temperature, the number of small colonies was highest when DNA was treated with T4 DNA polymerase at 50°C (Figure 3A). When plasmids were analyzed by restriction enzyme digestion, correct clones were found only in the small colonies (Figure 3C), suggesting that the insert affected growth of the host *E. coli* cells. Regular-

Figure 2. Effect of temperature of T4 DNA polymerase treatment on one-step sequence- and ligation-independent cloning (SLIC). (A) pGEX-5X was linearized by *Not*I digestion and mixed with *PARK7* insert prepared by PCR. T4 DNA polymerase was mixed and incubated at the indicated temperatures for 15, 30, and 60 s. Tubes were placed on ice, and DNA was transformed into competent *E. coli* cells. After incubating the plates for 16 h, colonies were counted. Data represent mean ± SD ($n = 3$). (B) Analysis of transformants by restriction enzyme digestion. (C) *Not*I-digested pGEX-5X and PCR amplified *PARK7* insert were mixed and treated with T4 DNA polymerase, as indicated. For Sample iv, T4 DNA polymerase was mixed on ice. After transformation, colonies were counted, and plasmids were analyzed by restriction enzyme digestion. *** $P < 0.001$.

The first-ever ergonomic serological pipet

Use with the VistaLab™ ali-Q™

Wobble-not™ Serological Pipet

Lose the Wobble, Lose the Drip!

Other serological pipets require jamming to get the pipet into your controller or risk wobble and dripping.

- Increase your accuracy and precision with Wobble-not™.
- Less fatigue due to the Wobble-not™ ergonomic design.
- Use with any pipet controller.

For the ideal pipetting system, use with **VistaLab ali-Q** aliquoting pipet controller.

Get your free sample pack
vistalab.com/wobble-not

VistaLab™
TECHNOLOGIES
Think Differently. Feel Better.

vistalab.com/wobble-not • +1 914-244-6226
2 Geneva Road, Brewster NY 10509

D

	Positive colonies / small colonies analyzed				
	25°C	37°C	50°C	65°C	75°C
15 s	2/4	4/4	4/4	4/4	3/4
30 s	4/4	4/4	4/4	4/4	3/4

Figure 3. One-step sequence- and ligation-independent cloning (SLIC) with a difficult cloning insert. (A) pUC118-HMG was linearized by *Bam*HI digestion and mixed with Ton_0858 prepared by PCR. T4 DNA polymerase was added, and samples were incubated at the indicated temperatures for 15 or 30 s. Tubes were placed on ice, and DNA was transformed into competent *E. coli* cells. After incubating plates for 16 h, regular-sized colonies were counted. Small colonies were counted after incubating plates for another 2 h at 37°C. Data represent mean $\pm$ SD ($n = 4$). (B) Representative picture of the colonies at 18 h. (C) Analysis of transformants by restriction enzyme digestion. Positive clones were obtained only from small colonies (Lanes 4–6 and 10). (D) Summary of the analysis of the small colonies by restriction enzyme digestion.

sized clones contained vector that either self-ligated after *Bam*HI-digestion or was uncut, with the number of regular-sized colonies similar to when vector alone was treated with T4 DNA polymerase (Figure 2C). When 4 small colonies from each temperature were analyzed, all conditions except 25°C for 15 s and 75°C for any period tested showed 100% cloning efficiency (Figure 3D). However, if the insert did not affect the growth of the host, and the colonies were therefore all the same size, the probability of picking correct clones would have been ~70% at 50°C for 30 s and approximately 50% for other conditions. This indicates that higher temperatures with shorter incubations (50°C for 30 s) are especially useful for cloning inserts with low cloning efficiency, consistent with the results shown in Figures 1 and 2. Sequencing of positive clones taken from each temperature for 30 s confirmed there were no mismatches within or at either side of the annealing sites during cloning. It is worth mentioning that if regular-sized colonies do not have the expected inserts, smaller and slower-growing colonies should be analyzed.

Here, we hypothesized that SLIC efficiency might be impaired by secondary structures formed in the ssDNA regions exposed at the ends of vectors and inserts by T4 DNA polymerase. Reducing the duration and increasing the temperature of T4 DNA polymerase treatment, both of which reduce secondary structure, improved SLIC efficiency for three different inserts. Furthermore, we suggest that 50°C for 30 s is an optimal SLIC condition for inserts with homology lengths <20 bases. This reduced incubation time should also improve the cloning perfor-

mance of smaller DNA fragments by one-step SLIC. Finally, our results also provide a new means of utilizing mesophilic T4 DNA polymerase at higher temperatures.

Author contributions

M.N.I., K.W.L., H.-S.Y., S.H.L., and H.C.J. performed the experiments. J.H.L. and J.Y.J. conceived the study. M.N.I. and J.Y.J. wrote the paper.

Acknowledgments

This study was supported by the in-house program (PE99514) from Korea Institute of Ocean Science and Technology (KIOST), and a Development of Biomedical Materials Based on Marine Proteins grant (PM60120) from the Ministry of Oceans and Fisheries in the Republic of Korea.

Competing interests

The authors declare no competing interests.

References

1. Li, M.Z. and S.J. Elledge. 2007. Harnessing homologous recombination in vitro to generate recombinant DNA via SLIC. *Nat. Methods* 4:251-256.
2. Gibson, D.G., L. Young, R.Y. Chuang, J.C. Venter, C.A. Hutchison 3rd, and H.O. Smith. 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat. Methods* 6:343-345.
3. Jeong, J.Y., H.S. Yim, J.Y. Ryu, H.S. Lee, J.H. Lee, D.S. Seen, and S.G. Kang. 2012. One-step sequence- and ligation-independent cloning as a rapid and versatile cloning method for functional genomics studies. *Appl. Environ. Microbiol.* 78:5440-5443.
4. Quan, J. and J. Tian. 2011. Circular polymerase extension cloning for high-throughput cloning of complex and combinatorial DNA libraries. *Nat. Protoc.* 6:242-251.
5. Bryksin, A.V. and I. Matsumura. 2010. Overlap extension PCR cloning: a simple and reliable way to create recombinant plasmids. *Biotechniques* 48:463-465.
6. de Kok, S., L.H. Stanton, T. Slaby, M. Durot, V.F. Holmes, K.G. Patel, D. Platt, E.B. Shapland, et al. 2014. Rapid and reliable DNA assembly via ligase cycling reaction. *ACS Synth. Biol.* 3:97-106.
7. Aslanidis, C. and P.J. de Jong. 1990. Ligation-independent cloning of PCR products (LIC-PCR). *Nucleic Acids Res.* 18:6069-6074.
8. Stevenson, J., J.R. Krycer, L. Phan, and A.J. Brown. 2013. A practical comparison of ligation-independent cloning techniques. *PLoS One* 8:e83888.
9. Rittié, L. and B. Perbal. 2008. Enzymes used in molecular biology: a useful guide. *J. Cell Commun. Signal.* 2:25-45.
10. Koskela, E.V. and A.D. Frey. 2015. Homologous recombinatorial cloning without the creation of single-stranded ends: exonuclease and ligation-independent cloning (ELIC). *Mol. Biotechnol.* 57:233-240.

Received 19 March 2017; accepted 25 July 2017.

Address correspondence to Jung-Hyun Lee or Jae-Yeon Jeong. Marine Biotechnology Research Center, Korea Institute of Ocean Science and Technology, Haeamro 787, Ansan 15627, Republic of Korea. E-mail: jlee@kiost.ac.kr or jeongjy@gmail.com

To purchase reprints of this article, contact: biotechniques@fosterprinting.com

NEW Fast, Controllable LED Illumination for Fura-2 Ratiometric Calcium Imaging

For more information on how CoolLED products can help you, contact us now:

t: +44 (0)1264 323040 (Worldwide)
1-800-877-0128 (USA/Canada)

w: www.CoolLED.com
e: info@CoolLED.com

www.CoolLED.com